Cloning, Sequence Analysis, and Expression of a cDNA Encoding a Plastid-Localized Heat Shock Protein in Maize[1]

Jorge Nieto-Sotelo[2], Elizabeth Vierling, and Tuan-Hua David Ho*

Plant Biology Program, Department of Biology, Division of Biology and Biomedical Sciences, Washington University, St. Louis, Missouri 63130 (J.N.-S., T.-H.D.H.); and Department of Biochemistry, University of Arizona, Tucson, Arizona 85721 (E.V.)

ABSTRACT

We have cloned and characterized a cDNA encoding a maize (*Zea mays* L.) heat shock protein (HSP), HSP26. The mRNA of HSP26 is present as a single mRNA species of 1.1 kilobase pairs in size and is detectable when maize seedlings are treated at 40°C but not at 28°C. Accumulation of HSP26 mRNA was detected after 10 minutes of incubation at 40°C, reaching the maximum level after 1 hour. Comparison of the deduced amino acid sequence of maize HSP26 to other HSPs indicated a strong homology to the sequences of two nuclear encoded HSPs that are transported into the chloroplasts during heat shock: pea HSP21 and soybean HSP22. Maize HSP26 was also found to cross-react with anti-pea chloroplast HSP21 antibodies. Because of the sequence homology between maize HSP26, soybean HSP22, and pea HSP21, *in vitro* chloroplast protein import experiments were conducted. The *in vitro* synthesized maize HSP26 is specifically imported to the soluble fraction of the chloroplast and processed to a smaller polypeptide. The sequence homology and antibody cross-reactivity between maize HSP26 and pea HSP21 have allowed us to conclude that maize HSP26 is a nuclear-encoded, plastid-localized protein in maize.

When living organisms are exposed to temperatures above their optimum for growth, a dramatic change in their pattern of gene expression is elicited (15). Transcription of a small set of genes referred to as heat shock genes is enhanced. These new mRNAs are translated into HSPs.[3] This response has been observed in species belonging to every major group of living organisms so far examined including bacteria, protozoa, fungi, algae, higher plants, invertebrates, and vertebrates. Studies of yeast strains carrying gene disruptions in HSP70 encoding genes (29) and in *Escherichia coli* strains carrying a mutation on the high temperature production regulating gene

(htpR) (30) have established that HSPs are necessary for survival at high temperatures. The function of some HSPs has already been determined in certain species: an isoform of lysyl tRNA synthetase in *E. coli* (9), an ATP-dependent protease in *E. coli* (19), ubiquitin in chicken embryo fibroblasts (4), and an isoprotein of enolase in yeast (12). Recently, it has been suggested that three classes of HSPs in eukaryotes, HSP90, HSP70 and GroEL, may function as 'unfoldases' or 'molecular chaperones' required for the correct folding or assembly of certain protein structures (15).

The response of higher plants to temperature stress is similar to that of other eukaryotes. However, a unique feature of the heat shock response in plants is that they also produce nuclear-encoded HSPs which localize to chloroplasts (14, 26). These chloroplast HSPs are homologous to low molecular mass HSPs found in the cytoplasm of plants and other eukaryotes (27).

We have been studying the heat shock response in maize with an interest in further characterizing the major HSPs in this organism. We have observed that a major 29 kD HSP in maize, which is seen by *in vitro* translation of heat shock poly(A)+ RNA, is not present in plants labeled *in vivo* (6). To investigate the regulation of the 29 kD HSP and to provide further information about low mol wt HSPs in plants, a cDNA clone encoding this HSP has been obtained. In the present paper we describe the characterization of this cDNA and its expression during heat shock. The predicted amino acid sequence indicates that the protein is homologous to nuclear-encoded, chloroplast-localized HSPs from pea and soybean (27). The product of this maize HSP cDNA could also be imported into chloroplasts *in vitro*, providing further evidence that this protein localizes to chloroplasts *in vivo*.

MATERIALS AND METHODS

RNA Isolation

Total RNA was isolated from 3-d-old maize seedlings (*Zea mays* L., Hybrid 222, Crow's Hybrid Corn Co., Milford, IL) grown at 28°C in the dark. All manipulations, from seed germination to temperature treatments, were carried out under aseptic conditions. Roots and coleoptiles were sectioned into 2.5 cm pieces and preincubated in flasks containing 20 mM Na succinate (pH 5.0), 0.1 mM CaCl₂ for 3 h at 28°C prior to further treatment at 28 or 40°C for the indicated times. After treatment, plant material was frozen under liquid

N_2 and kept at $-70°C$ until RNA isolation. Total RNA was isolated as described (21). Poly(A)$^+$ RNA was selected twice by oligo (dT) cellulose chromatography as described (16) except that the binding buffer was 0.5 M LiCl, 10 mM Tris-HCl (pH 7.4), and 0.5% SDS, and the elution buffer was 10 mM Tris-HCl, (pH 7.4), 0.5% SDS.

Size Fractionation of poly(A)$^+$ RNA

Twenty-five μg of poly(A)$^+$ RNA isolated from 3-d-old seedlings which had been heat-shocked for 2 h at $40°C$ were size-fractionated by sedimentation velocity in sucrose gradients. The RNA sample was diluted in 10 mM Tris-HCl (pH 7.4), 10 mM LiCl, 1 mM EDTA, 10% DMSO, 4% formamide, incubated at $60°C$ for 10 min and quenched on ice prior to loading onto a 5 to 20% linear sucrose gradient. The sucrose gradient was prepared in 20 mM Tris-HCl (pH 8.0), 100 mM LiCl, 5 mM EDTA, and kept at $4°C$ for 4 h prior to loading. The gradient was centrifuged in ultraclear tubes in a SW41 rotor (Beckman, Palo Alto, CA) at 32,000 rpm (130,000 g at r_{av}) at $4°C$ for 18 h. Fractions were collected with an ISCO fraction collector coupled to a UV monitor to determine $A_{280 \, mm}$. Each fraction was adjusted to 0.3 M Na acetate and precipitated with 2 volumes of ethanol at $-20°C$. Final pellets were resuspended in 20 μL of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 5 μL aliquots of each fraction were translated *in vitro* in nuclease treated-rabbit reticulocyte lysates (Promega, Madison, WI).

Construction and Screening of Heat Shock cDNA Library

A heat shock cDNA library enriched for clones encoding intermediate size HSPs (20–40 kD) was constructed by the method of Huynh *et al.* (11) using poly(A)$^+$ RNA (approximately 0.75 μg) from the size fractions enriched with these mRNAs. cDNAs above 300 bp were ligated to *Eco*RI digested λgt10 and packaged *in vitro* (Stratagene, San Diego, CA). *E. coli* C600 hfl$^-$ was used as the host strain for growth of recombinant phage. A total of 5.1 × 10^5 recombinants was obtained. Recombinants were screened by differential hybridization with single-stranded cDNA probes complementary to mRNA from either control (28°C) or heat-shocked (40°C) maize seedlings incubated for 2 h. Confirmed positives were plaque-purified, and the cDNA inserts were subcloned in both orientations into the *Eco*RI site of the Blue Script KS (+) plasmid (Stratagene).

DNA Sequencing and Sequence Analysis

The nucleotide sequence of the cDNA was determined by the dideoxy method of Sanger *et al.* (22) using [^{35}S]dATP α-S (specific activity: >1000 Ci mmol^{-1}, 40 TBq; NEN, Boston, MA) and 'Sequenase' (USB, Cleveland, OH). Reaction products were run on buffer-gradient gels (3). DNA sequence analyses were performed on a VAX computer using programs of the Genetics Computer Group Sequence Analysis Software Package.

Northern Hybridization

RNA samples were electrophoresed in agarose-formaldehyde gels as described (16). A ladder of RNA size standards

(BRL, Bethesda, MD) was run to estimate the RNA size. After blotting RNA to GeneScreen (Dupont, Wilmington, DE) RNA was UV cross-linked to the membrane for 5 min. Prehybridization and hybridization were performed as described (5). ^{32}P-Labeled DNA probes were prepared by the random primer method (8).

In Vitro Import of Proteins into Isolated Chloroplasts

Import of *in vitro* translation products into isolated, intact pea (*Pisum sativum*) chloroplasts was performed as described previously (26). Pea chloroplasts have been shown to faithfully import and process precursors from diverse higher plant species including maize. [^{35}S]Methionine labeled proteins for import were produced by *in vitro* translation of control or heat shock poly(A)$^+$ RNA isolated from leaves of 14-d-old maize plants (28), or by *in vitro* translation of transcripts produced from the cloned maize cDNAs. RNA was translated in reticulocyte lysates according to the supplier (BRL) using 1 $\mu Ci/\mu l$ of [^{35}S]methionine (>1,000 Ci mmol^{-1}, 40.0 TBq mmol^{-1}; NEN). Intact chloroplasts from 9-d-old pea plants were purified on Percoll gradients, and incubated for 30 min in the light in the presence of the [^{35}S]methionine labeled proteins. Following the import reaction, chloroplasts were treated on ice for 30 min with trypsin and chymotrypsin (20 $\mu g/mL$) to remove proteins nonspecifically bound to the chloroplast envelope. Proteolysis was stopped by the addition of protease inhibitors (1 mM PMSF, 1 mM ϵ-aminocaproic acid, 5 mM benzamidine) and intact chloroplasts were reisolated and lysed in 20 mM Hepes (pH 8) plus protease inhibitors. Thylakoid and envelope membranes were pelleted by centrifugation at 12,000g for 30 min. The soluble and membrane fractions were either analyzed directly by gel electrophoresis on 10 to 16% gradient SDS gels, or used for immunoprecipitations prior to electrophoretic analysis.

Immunoprecipitation

For immunoprecipitation, rabbit antibodies against the carboxyl terminal segment of pea chloroplast HSP21 were utilized. Production and specificity of these antibodies have been described by Vierling *et al.* (28). Immunoprecipitations from reticulocyte *in vitro* translation reactions or from chloroplast fractions of import reactions were performed according to Anderson and Blobel (1).

RESULTS

Comparison of *in vivo* and *in vitro* Synthesized Proteins in Heat Shocked Maize Seedlings

It has been well documented that maize seedlings synthesize HSPs under heat shock treatment (40°C) (7). The profile of *in vitro* translation products of RNA isolated from heat shock tissue, in general, corresponds to the profile of proteins synthesized *in vivo* during heat stress (Fig. 1). A major exception is the relative synthesis *in vitro* of a HSP with an apparent size of 29 kD (Fig. 1, lanes 2 and 6). This HSP is either absent or synthesized at very low levels *in vivo* (Fig. 1, lane 2). The low level of this HSP observed *in vivo* may be due to preferential translation of its mRNA *in vitro*, a high degree of
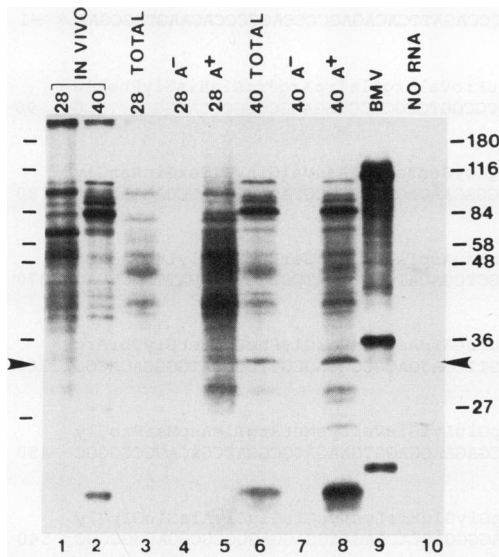
Figure 1. Comparison of the proteins translated *in vivo* or *in vitro* in maize during heat shock. The fluorogram shows proteins separated by electrophoresis in 11.5% polyacrylamide-SDS gels. Lanes 1 and 2, protein synthesis pattern in excised maize root tips from 2-d-old seedlings that were labeled with [^{35}S]methionine for 2 h *in vivo* at either 28°C (lane 1) or 40°C (lane 2). Lanes 3 to 8, pattern of proteins synthesized *in vitro* using total, poly(A)$^-$ or poly(A)$^+$ RNA isolated from maize roots and coleoptiles after treatment for 2 h at 28°C (lanes 3–5) or 40°C (lanes 6–8). Lane 9, BMV RNA; lane 10, no RNA control. Protein molecular mass markers are shown in the right margin. Arrows indicate position of the HSP with an apparent size of 29 kD discussed in the text.

turnover of the protein *in vivo*, or post-translational processing of the protein to a different mature form. To investigate these possibilities, we have cloned and characterized this particular HSP.

Isolation and Characterization of HSP cDNAs

A heat shock cDNA library was constructed and screened as described under "Materials and Methods." Translation *in vitro* of the mRNA selected by two of the 11 clones we have isolated, clone 241 (0.5 kb) and clone 17 (1.0 kb), demonstrated that they encoded the 29 kD HSP (data not shown). The larger clone, clone 17, was selected for further characterization. This cDNA hybridized to a 1.1 kb mRNA detected only in heat shocked maize seedlings. The size of the mRNA and the length of the cDNA insert in clone 17 suggested that the cDNA sequence was close to full length. This is confirmed by the coupled *in vitro* transcription and translation experiment with clone 17 yielding a prominent polypeptide of 29 kD (data not shown).

Time Course of Induction of HSP mRNAs

To demonstrate further that clone 17 encoded an HSP, clone 17 was used to probe Northern blots of mRNA isolated from heat-treated maize seedlings. Total RNA was isolated from 3-d-old etiolated seedlings (roots and coleoptiles) that had been treated for various lengths of time at either 28 or

40°C (Fig. 2). Northern blots probed with either pMON9501, a genomic clone encoding maize HSP70 (21), or with clone 17, showed that 10 min of treatment at 40°C caused an increase in the levels of both HSP70 (Fig. 2, panel B) and clone 17 mRNA (Fig. 2, panel C). Clone NcN155, an unidentified cDNA from maize, was used to probe the same RNAs (Fig. 2, panel A) to demonstrate the presence of intact RNA in the control samples. While HSP70 showed a low basal level of mRNA at 28°C, clone 17 mRNA was undetectable at this temperature. Maximum levels of mRNA for HSP70 and clone 17 were achieved after 1 h of heat shock although with different kinetics. HSP70 mRNA levels persisted for the 5 h heat shock treatment, whereas clone 17 mRNA levels declined between 1 and 5 h of heat shock. After 5 h of heat shock a very low molecular mass RNA homologous to the clone 17 probe accumulated. The identity of this band is unclear, but it may represent a specific mRNA degradation product. In total, these data confirm that clone 17 encodes an HSP.

Nucleotide Sequence of Clone 17 and Its Predicted Amino Acid Sequence

The entire cDNA insert (950 nucleotides without the poly[A] segment) was determined on both strands and is
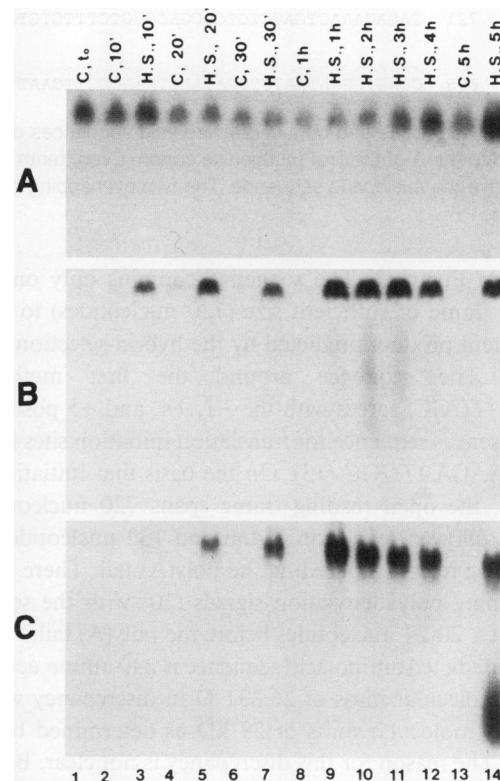

Figure 2. Time course of expression of clone 17 mRNA in control and heat shocked maize seedlings. Northern blot containing total RNA isolated from 3-d-old etiolated seedlings (roots and coleoptiles) treated for the times indicated at 28°C (C) (lanes 1, 2, 4, 6, 8, and 13) or 40°C (H.S.) (lanes 3, 5, 7, 9–12, and 14). Top panel (A), probing with NcN155; middle panel (B), probing with the HSP70 clone, pMon9501; lower panel (C), probing with clone 17.

```
-90  TTTTTTACCAAATCCGGACAGTCTTGAGCTCTCTCAGTCTCAGCATTTCAGTTCCCAGATTCACAGAGCCCCAGCCCCACAAGTAGCGAA   -1


     MetAlaAlaAlaProPheAlaIleAlaGlyArgLeuSerProValAlaArgLeuProValArgAlaTrpArgProAlaHisGlyPheAla
  1  ATGGCAGCTGCTCCGTTCGCGATTGCCGGCCGGCTCTCCCCCGTTGCGCGCCTCCCGGTCCGCGCCTGGAGGCCGGCGCACGGGTTTGCG   90


     SerSerGlyArgAlaArgSerLeuAlaValAlaSerAlaAlaGlnGluAsnArgAspAsnSerValAspValGlnValSerGlnAsnGly
 91  TCGTCCGGGAGAGCCCGCTCGCTCGCCGTGGCATCCGCGGCGCAGGAGAACAGGGACAACAGTGTCGACGTACAGGTTAGCCAGAACGGC   180


     GlyAsnArgGlnGlnGlyAsnAlaValGlnArgArgProArgArgAlaThrAlaLeuAspIleSerProSerProPheGlyLeuValAsp
181  GGCAACAGGCAGCAGGGCAACGCCGTGCAGCGCCGCCCGCGCCGCGCGACAGCGCTCGACATCTCCCCATCCCCGTTCGGATTAGTTGAC   270


     ProMetSerProMetArgThrMetArgGlnMetLeuAspThrMetAspArgLeuPheAspAspAlaValGlyPheProMetGlyThrArg
271  CCGATGTCGCCGATGCGGACGATGCGGCAGATGCTGGACACGATGGACAGGCTGTTCGACGACGCCGTGGGGTTCCCCATGGGGACACGG   360


     ArgSerProAlaThrThrGlyAspValArgLeuProTrpAspIleValGluAspGluLysGluValLysMetArgIleAspMetProGly
361  AGGTCCCCGGCGACCACCGGCGACGTACGCCTGCCGTGGGACATCGTGGAGGACGAGAAGGAGGTGAAGATGCGGATCGACATGCCGGGC   450


     LeuAlaArgAspGluValLysValMetValGluAspAspThrLeuValIleArgGlyGluHisLysLysGluGluGlyAlaGluGlyGly
451  CTCGCGCGCGACGAGGTGAAGGTGATGGTGGAGGACGACACGCTGGTCATCCGAGGGGAGCACAAGAAGGAGGAGGGCGCGGAGGGCGGC   540


     SerGlyGlyAspGlyAspGlyTrpTrpLysGlnArgSerValSerSerTyrAspMetArgLeuAlaLeuProAspGluCysAspLysSer
541  AGCGGCGGCGACGGCGACGGGTGGTGGAAGCAGCGCAGCGTGAGCTCCTACGACATGCGGCTGGCGCTGCCGGATGAGTGCGACAAGAGC   630


     LysValArgAlaGluLeuLysAsnGlyValLeuLeuValThrValProLysThrGluValGluArgLysValIleAspValGlnValGln
631  AAGGTGCGGGCCGAGCTCAAGAACGGCGTGCTCCTCGTCACCGTGCCCAAGACCGAGGTGGAGCGCAAGGTCATCGACGTGCAGGTCCAG   720


     End
721  TAGACAAACTGAGCTGTCACGACGGGTCTTTGTGTAGCACTAGCGTGGGTTGGGCTCTTCGCGCCGACCGTATCAGTACTCGTGACCCAA   810


805  CGAGTGGAATAATGTGAATAAATAAACGCTTCAATTCTGCTATTCCAAAAAA
```

Figure 3. Nucleotide and deduced amino acid sequences of clone 17. Negative numbers refer to the 5'-noncoding region. Nucleotide +1 was assigned to the A of the first methionine codon. Every tenth nucleotide is denoted by a '·'; amino acids are indicated in the standard three letter code above the nucleotide sequence. The two overlapping polyadenylation signals, AATAAA, are underlined.

shown in Figure 3. The sequence contains only one open reading frame of sufficient size (720 nucleotides) to encode the protein product predicted by the hybrid-selection experiments. The sequence around the first methionine, CGAA*ATG*GC, agrees with the −1, +4, and +5 positions of the consensus sequence for translation initiation sites in plant genes, AACA*ATG*GC (13). On the basis that initiation is at site +1, the open reading frame spans 720 nucleotides in length, followed by a stop codon and 133 nucleotides of 3' noncoding region proceeding the poly(A) tail. There are two overlapping polyadenylation signals (20) with the sequence AATAAA 20-24 nucleotides before the poly(A) tail.

The predicted amino acid sequence is 240 amino acids long with a molecular mass of 26,331 D in discrepancy with the apparent molecular mass of 29 kD as determined by SDS-PAGE. The reason for this discrepancy is not clear. Based on the predicted molecular mass, we have designated this HSP as HSP26 and clone 17 is now referred to as pZmHSP26. HSP26 has no potential glycosylation sites (Asn-X-Thr/Ser) (10). The hydropathy profile of HSP26 reveals that for the most part, the protein is hydrophilic and no apparent membrane spanning segments are present.

Comparison of pZmHSP26 to Other Heat Shock Protein Encoding Genes

A comparison of the pZmHSP26 coding region with the coding regions of other HSP genes revealed a high percentage of similarity to the recently determined sequences of soybean HSP22 and pea HSP21 (27). These HSPs are synthesized in the cytosol as precursor proteins which are processed to their mature size during import into the chloroplasts. As shown in Figure 4, at the amino acid level the similarity between maize HSP26 and pea HSP21 is low near the amino terminus (the first 55 amino acids) and increases as the comparison progresses toward the carboxyl terminus. This lack of similarity near the amino terminus is not unexpected because the first 45 to 46 amino acids of pea HSP21 have been suggested to represent the transit peptide. Transit peptides typically show little sequence similarity even between homologous proteins (23). Similarity of maize HSP26 to pea HSP21 and soybean HSP22 is significant throughout the sequence representing the processed, mature segment of the chloroplast HSPs. The sequence similarity is particularly striking over the carboxyl terminal 46 amino acids, 37 of 46 amino acids are identical to soybean HSP22 and 31 of 46 to pea HSP21. Many of the differences represent conservative replacements such as Ile for

```
         1                                              50
ZmHSP26  ........MA AAPFAIAGRL SPVARLP..V RAWRPAHGFA SSGRARSLAV
GmHSP22  .......... .......... .......... .......... ..........
PsHSP21  MAQSVSLSTI *S*ILSQKPG *S*KST*PCM ASFPLRRQLP RLGLRNVR*Q
GmHSP34  .......... .......... .......... .......... ..........
GmHSP17  .......... .......... .......... .......... ..........
DmHSP26  .......... .......... .......... .......... .MSLST**SL

         51                                            100
ZmHSP26  ASAAQENRDN SVDVQVSQNG GNRQQGNAVQ RRPRRATALD ISPSPFGLVD
GmHSP22  ...GGD*K** **E*...*HV SKGD**T**G KK***.TAM* *..****IL*
PsHSP21  *GGDGD*K** **E*...HRV NKDD**T**E RK***SS... *DI****L*
GmHSP34  .......... .......... .......... .......... ..........
GmHSP17  .......... .......... .......... .......... .....MSLIP
DmHSP26  VDEL*DP*SP IYQLGLGLHP HS*YVLLPLG TQQ**SINGC PCA**ICPSS

         101                                           150
ZmHSP26  PMSPMRTMRQ MLDTMDRLFD DAVGFPMGTR RSPATTGDVR LPWDIVEDEK
GmHSP22  *W****S*** I******V*E *TMT**.... GRNIGG*EI* A****KDE*H
PsHSP21  *W****S*** *******I*E *AITI*.... GRNIGG*EI* V**E*KDE*H
GmHSP34  .......... .......... .......... .......... ..........
GmHSP17  SFFGG*RSSV FDPFSLDVW* PFKD**FPSS L*AENSAFVS TRV*WK*TPE
DmHSP26  *AGQV..... .......... ......LAL* *EM*NRNDIH W*ATAHVGKD

         151                                           200
ZmHSP26  EVKMRIDMPG LARDEVKVMV EDDTLVIRGE HKKEEGAEGG SGGDGDGWWK
GmHSP22  *IR**F**** **KED***S* ***M***K*G **S*QEH... ...G**DS*S
PsHSP21  *IR**F**** VSKED***S* ***V***KSD *RE*N*G*DC ........*S
GmHSP34  .......... .......... ...ILQ*S** RNV*KE.... .DKNDTWHRV
GmHSP17  AHVFKA*I** LKKE*V*LEI Q*GRVLQISG ERNVEKED.. ..KNDTWHRV
DmHSP26  GFQVCM*VAQ FKPS*LN*K* V*ASILVE*K *EERQDDH*. ..........

         201                                           250
ZmHSP26  QRSVSSYDMR LALPDECDKS KVRAEL.KNG VLLVTVPKTE VERKVIDVQV
GmHSP22  S*TY****T* *K***N*E*D **K***.*** **YI*I**** **********
PsHSP21  RK*Y*C**T* *K***N*E*E **K***.*D* **YI*I***K I**T*****I
GmHSP34  E**SGKFMRS FR*^*NAKVD Q*K*SM.E** **T*****E* IKKPDVKAIE
GmHSP17  E**SGKLVR* FP.*^*AKVD Q*K`3!.F.** **T*****E* IKKPDVKAID
DmHSP26  .HIMGHFVR* YKV**GYKAE Q*VSQ*SSD* **T*SI**P* AVEDKSKERI

         251                            284
ZmHSP26  Q#....... .......... .......... .....
GmHSP22  *#....... .......... .......... .....
PsHSP21  *#....... .......... .......... .....
GmHSP34  ISG#..... .......... .......... ....
GmHSP17  ISG#..... .......... .......... ....
DmHSP26  IQIQQVGPAH LNVKANDSEV KGKENGAPNG KDK#
```

Figure 4. A comparison of the pZmHSP26 predicted amino acid sequence to other HSPs. Alignment of the predicted amino acid sequences of maize HSP26 (ZmHSP26), soybean chloroplast HSP22 (GmHSP22), pea chloroplast HSP21 (PsHSP21), soybean HSP34 (GmHSP34), soybean HSP17 (GmHSP17), and Drosophila HSP26 (DmHSP26). All sequences represent the complete coding region except soybean chloroplast HSP22 and HSP34. Identical matches to the ZmHSP26 sequence are identified by *s. Gaps (·s) are created for better matchings among the sequences. The underlined region denotes the most invariant sequencing among small molecular mass HSPs, aa$_1$-aa$_2$-Gly-aa$_3$-Leu-aa$_4$-aa$_5$-aa$_6$-aa$_7$-Pro-aa$_8$ (15).

Val. This region includes a small stretch of amino acids (No. 228-237 in Fig. 4), aa$_1$-aa$_2$-Gly-aa$_3$-Leu-aa$_4$-aa$_5$-aa$_6$-aa$_6$-Pro-aa$_8$, identified in all low molecular mass HSPs (15).

Two other regions of significant similarity between maize HSP26 and the pea and soybean chloroplast HSPs are evident from amino acid position 84-112 and 141-171 of the maize protein (No. 94-122 and 151-181 in Fig. 4). There are three apparent insertions in the maize sequence which occur between these two segments and the carboxyl terminal conserved region.

Table I shows the percent similarity of several HSPs, relative to maize HSP26. The values take into account both identical and conservative amino acid replacements. In general, maize HSP26 showed a higher percent of similarity to the low molecular mass plant HSPs than to Drosophila HSP27, 26, 23, or 22. Inspection of the alignment made in Figure 4 also shows that relative to Drosophila HSP26, maize HSP26 and pea HSP21 contain an additional 31-38 residues at the amino terminus, which further suggests the presence of a transit peptide in the latter proteins. Comparison of hydropathy profiles of Drosophila HSP26 with the maize and pea HSPs

also suggests overall structural similarity between these HSPs (data not shown). Taken together, these observations suggest that HSP26 is the maize homolog of the pea chloroplast HSP21.

Import and Processing of HSP26 by Isolated Chloroplasts

The sequence homology between maize HSP26 and the pea and soybean chloroplast HSPs strongly suggests that maize HSP26 is also localized to the chloroplast. To confirm that maize HSP26 could be imported and processed by chloroplasts, in vitro import experiments were performed. As reported previously (26), when [^{35}S]methionine labeled in vitro translation products of poly(A)$^+$ RNA from control or heat-stressed maize leaves were used for import into isolated pea chloroplasts, several polypeptides were recovered in the heat shock samples which were not seen in the controls (Fig. 5, lane 4 versus lane 3). The most abundant of these proteins is a polypeptide of approximately 24 kD. Several high molecular mass polypeptides (approximately 98, 70, and 57 kD) were also observed. An import reaction was then performed using polypeptides synthesized by in vitro translation of transcripts produced from pZmHSP26. In this experiment a polypeptide which comigrated with the 24 kD protein was recovered in the soluble fraction of the chloroplast. From these results, we conclude that HSP26 represents the precursor of the major low molecular mass chloroplast-localized HSP produced during heat shock of maize plants.

The homology of maize HSP26 and pea chloroplast HSP21 was further confirmed using antibodies raised against the carboxyl terminal segment of pea HSP21 (28). As shown previously, two polypeptides were immunoprecipitated from in vitro translation products of heat shock maize poly(A)$^+$ RNA using these antibodies (Fig. 5). The most abundant of these comigrated with the pZmHSP26 transcription-transla-

Table I. Percentage Similarity between Maize HSP26 and Other HSPs

HSP	Similarity	
	Protein	DNA
	%	
Soybean HSP22[a]	82	61
Pea HSP21[b]	79	56
Pea HSP21[c]	26	39
Pea HSP21[d]	71	52
Soybean HSP34[e]	53	35
Soybean HSP17	49	42
Drosophila HSP26	48	43
Drosophila HSP27	38	42
Drosophila HSP22	38	38
Soybean HSP26	37	38
Drosophila HSP23	37	41

[a] Comparison made with the available 181 carboxyl terminal amino acids of this protein.   [b] HSP21 protein region from amino acid 54 to 232 (representing the mature protein).   [c] From amino acid 1 to 53 (representing the transit peptide).   [d] HSP21 whole protein sequence from amino acid 1 to 232.   [e] Comparison made with the available 75 carboxyl terminal amino acid of HSP34.
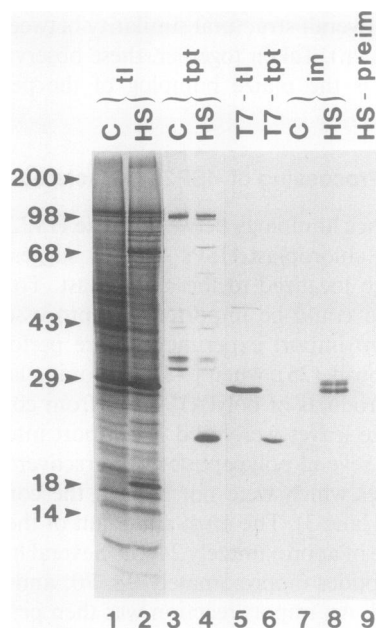
Figure 5. Import of maize HSP26 into isolated pea chloroplasts. Lane 1, *In vitro* translation products of control maize poly(A)$^+$ RNA; lane 2, as in lane 1 only using heat shock poly(A)$^+$ RNA; lane 3, proteins recovered in the soluble fraction of the chloroplast following import of the polypeptides shown in lane 1; lane 4, as for lane 3 following import of polypeptides shown in lane 2; lane 5, *in vitro* translation products of transcripts synthesized from the HSP26 cDNA; lane 6, proteins recovered in the soluble fraction of the chloroplast following import of the protein shown in lane 5; lane 7, proteins immunoprecipitated from translation products of control poly(A)$^+$ RNA using anti-pea HSP21 antibodies; lane 8, as in lane 7 only with translation products of heat shock poly(A)$^+$ RNA; lane 9, *in vitro* translation products of heat shock poly(A)$^+$ RNA immunoprecipitated using preimmune serum. Samples were separated on a 10 to 16% gradient gel and then subjected to fluorography. Positions of molecular mass markers are shown at the left. tl, *In vitro* translation products; tpt, proteins transported into isolated chloroplasts.

tion product indicating that the pea and maize proteins are antigenically related. The antibodies also recognized a second, slightly larger polypeptide than that encoded by pZmHSP26. The origin of this larger polypeptide is unclear. The pea antibodies were also used for immunoprecipitation of chloroplast fractions from the import of translation products of control or heat shock maize poly(A)$^+$ RNA. In the heat shock immunoprecipitate the 24 kD polypeptide was visible, but only after extended exposure of the autoradiograms (data not shown). It was previously shown that the pea antibodies weakly react with a 24 kD HSP which accumulates in heat stressed maize plants (28). In total, these data indicate that HSP26 is the maize homologue of chloroplast HSP21 from pea.

DISCUSSION

We have characterized a cDNA clone for a low molecular mass HSP from maize, HSP26, and determined that it encodes the complete amino acid sequence of a chloroplast-

localized HSP in this species. Northern analysis demonstrated that HSP26 mRNA is absent in control seedlings, but strongly induced by heat and that the size of the pZmHSP26 cDNA is similar to the mRNA size *in vivo*. *In vitro* translation of hybrid-selected mRNAs or of *in vitro* transcripts from pZmHSP26 yielded identical polypeptides with an apparent molecular mass of 29 kD. DNA sequence analysis revealed pZmHSP26 has a complete open reading frame for a 26,331 D protein with high homology to low molecular mass HSPs from plants and other eukaryotes. The discrepancy between the predicted molecular mass and the molecular mass determined by SDS gel electrophoresis is typical of small HSPs (25).

Several lines of evidence indicate that HSP26 is localized to chloroplasts, or plastids, in maize. The amino acid sequence of HSP26 showed the highest homology to soybean HSP22 and pea HSP21 which are nuclear-encoded, chloroplast HSPs in these species (27). The amino terminal 45 to 55 amino acids of HSP26 have characteristics typical for chloroplast transit peptides including high Ser content, multiple basic residues and complete absence of acidic residues (23). As would be expected, when HSP26 is incubated with isolated chloroplasts *in vitro* it is imported and processed to a smaller size (24 kD on a 10–16% gel) by these organelles. Antiserum specific for pea chloroplast HSP21 recognized both the precursor and mature forms of maize HSP26. Finally, the fact that HSP26 would be processed *in vivo* during localization to the chloroplast is consistent with our observation that *in vivo* labeled seedlings do not produce a major polypeptide the size of the primary translation product of HSP26.

Characteristics of HSP26 are in agreement with previous observations of the major small chloroplast HSP in maize (26, 28). Vierling *et al.* (28) showed that antibodies generated against the carboxyl-terminal portion of pea chloroplast HSP21 recognized a maize HSP with an apparent size of 29 kD. When the pea antibodies were used to probe maize plants heat stressed *in vivo*, a 24 kD protein was shown to accumulate and there was no detectable accumulation of the 29 kD form. The 24 kD protein had also been identified as a maize chloroplast HSP by *in vitro* chloroplast import studies (26). Furthermore, it was found that antibodies generated against an amino terminal segment of pea HSP21 (representing approximately the first 50 amino acids of the processed, mature protein), did not react with the maize protein (28). Sequence comparison of the pea and maize proteins explains this difference in cross-reactivity of the amino and the carboxyl-terminal antibodies. Although there is extensive homology between the carboxyl-terminal regions of maize HSP26 and pea HSP21, their amino-terminal segments have only limited homology. In total these observations strongly support our conclusion that HSP26 is a major, chloroplast-localized HSP in maize.

Including maize HSP26, only two DNA sequences are available for low molecular mass HSPs from monocotyledonous plants. Recently, the sequence of a 150 amino acid HSP from wheat has been determined (17). Analysis of the wheat HSP indicates it has significantly higher homology to cytoplasmic HSPs of pea and soybean than to the chloroplast HSPs. Interestingly, comparison of the monocot and dicot

cytoplasmic HSPs shows the same pattern of homology we find between the maize and dicot chloroplast HSPs. There is extensive identity in the carboxyl-terminal two-thirds of the proteins, and limited homology in the remaining amino-terminal segment. The conserved carboxyl-terminal regions are thought to be important for low molecular mass HSP function and structure (15).

Maize pZmHSP26 was isolated from a cDNA library generated with poly(A)$^+$ RNA isolated from dark grown seedling tissues which lack functional chloroplasts. Kloppstetch et al. (14) and Vierling et al. (26) have also observed chloroplast HSP mRNAs in nonphotosynthetic tissues. It is interesting that although HSP26 mRNA appears to be abundant in these maize seedlings, there does not appear to be a comparable level of synthesis of the mature, 24 kD protein in vivo. Of 11 Met residues in HSP26, only one would be removed during import into the chloroplast. Therefore, the difference in apparent levels of available precursor compared to mature product cannot be due to differences in the protein's specific activity. In pea, we have observed that HSP21 mRNA accumulates to similar levels in leaf and root tissues, but that although the mature protein accumulates in roots, it is much less abundant than in leaves (E Vierling, Q Chen, unpublished data). Whether chloroplast HSP accumulation is in some way controlled by the number and function of plastids within a plant cell remains to be investigated.

The physiological role of the low molecular mass HSP within the chloroplast is not known. Schuster et al. (24) have suggested that these HSPs play a role in protecting the photosynthetic apparatus from photoinhibition during heat stress. Sensitivity of chloroplast functions to high temperature is well documented (2). In previous studies we have shown that maize plastids and mitochondria do not synthesize their own HSPs (18). Therefore, these organelles may be dependent on the import of nuclear-encoded HSPs for protection from stress. In addition, the synthesis of chloroplast HSPs in nonphotosynthetic tissues suggests that HSP function is not limited to protecting photosynthesis.

Homology between the chloroplast and cytoplasmic HSPs indicates these proteins may act through similar molecular mechanisms. At present, the function of low molecular mass cytoplasmic HSPs is not understood (15). These cytoplasmic HSPs form what appears to be homo-oligomeric, 10-20S particles in many eukaryotes (15). Preliminary data suggest that the chloroplast HSPs are also found in large particles (E Vierling, Q Chen, unpublished observations). It will be interesting to determine if the low molecular mass HSPs have functions similar to the 'unfoldase' and 'chaperone' functions proposed for other major classes of HSPs.

ACKNOWLEDGMENTS

LITERATURE CITED

1. Anderson DJ, Blobel G (1983) Immunoprecipitation of proteins from cell-free translation. Methods Enzymol 96: 111-120

2. Berry J, Björkman O (1980) Photosynthetic response and adaptation to temperature in higher plants. Annu Rev Plant Physiol 31: 491-543
3. Biggin MD, Gibson TJ, Hong GF (1983) Buffer gradient gels and ^{35}S label as an aid to rapid DNA sequence determination. Proc Natl Acad Sci USA 80: 3963-3965
4. Bond U, Schlesinger MJ (1985) Ubiquitin is a heat shock protein in chicken embryo fibroblast. Mol Cell Biol 5: 949-956
5. Church GM, Gilbert W 91984) Genomic sequencing. Proc Natl Acad Sci USA 81: 1991-1995
6. Cooper P (1985) The Heat Shock Response in Maize: Their Induction, Cellular Location and Potential Function. PhD. Thesis, University of Illinois at Urbana-Champaign, Urbana, IL
7. Cooper P, Ho THD (1987) Intracellular localization of heat shock proteins in maize. Plant Physiol 84: 1197-1203
8. Feinberg A, Vogelstein B (1983) Addendum: A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 132: 6-13
9. Hirshfield IN, Bloch PL, Van Bogelen RA, Niedhardt FC (1981) Multiple forms of lysyl-transfer ribonucleic acid synthetase in Escherichia coli. J Bacteriol 146: 345-351
10. Hubbard SC, Ivatt RJ (1981) Synthesis and processing of asparagine-linked oligosaccharides. Annu Rev Biochem 50: 555-583
11. Huynh TV, Young RA, Davis RW (1985) Constructing and screening cDNA libraries in λgt10 and λgy11. In DM Glover ed, DNA Cloning—A Practical Approach, Vol I. IRL Press, Oxford. pp 49-78
12. Iida H, Yahara I (1985) Yeast heat-shock protein of M_r 48,000 is an isoprotein of enolase. Nature 315: 688-690
13. Joshi CP (1987) An inspection of the domain between putative TATA box and translation start site in 79 plant genes. Nucleic Acids Res 15: 6643-6653
14. Kloppstech K, Meyer G, Schuster G, Ohad I (1985) Synthesis, transport and localization of nuclear coded 22-kd heat-shock protein in the chloroplast membranes of peas and Chlamydomonas. EMBO J 1: 1901-1909
15. Lindquist S, Craig EA (1988) The heat-shock proteins. Annu Rev Gent 22: 631-677
16. Maniatis T, Frisch EF, Sambrook J (1982) Molecular Cloning—A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
17. McElwain EF, Spiker S (1989) A wheat cDNA which is homologous to the 17kD heat-shock protein gene family of soybean. Nucleic Acid Res 17: 1764.
18. Nieto-Sotelo J, Ho THD (1987) Absence of heat shock protein synthesis in isolated mitochondria and plastids from maize. J Biol Chem 262: 12288-12292
19. Philips TA, VanBogelen RA, Niedhardt FC (1984) Lon gene product of Escherichia coli is a heat-shock protein. J Bacteriol 159: 283-287
20. Proudfoot N, Brownlee G (1981) Sequence at the 3'end of globin mRNA shows homology with immunoglobulin light chain mRNA. Nature 252: 359-362
21. Rochester DE, Winter JA, Shah DM (1986) The structure and expression of maize genes encoding the major heat shock protein, hsp 70. EMBO J 5: 451-458
22. Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74: 5463-5467
23. Schmidt GW, Mishkind ML (1986) The transport of proteins into chloroplasts. Annu Rev Biochem 55: 879-912
24. Schuster G, Even D, Kloppstech K, Ohad I (1988) Evidence for protection by heat-shock proteins against photoinhibition during heat-shock EMBO J 7: 1-6.
25. Southgate R, Ayme A, Voellmy R (1983) Nucleotide sequence

analysis of the Drosophila small heat shock gene cluster at locus 67B. J Mol Biol **165**: 35–57

26. **Vierling E, Mishkind ML, Schmidt GW, Key JL** (1986) Specific heat-shock proteins are transported into chloroplasts. Proc Natl Acad Sci USA **83**: 361–365

27. **Vierling E, Nagao RT, DeRocher AE, Harris LM** (1988) A heat shock protein localized to chloroplasts is a member of a eukaryotic superfamily of heat shock proteins. EMBO J **7**: 575–581

28. **Vierling E, Harris LM, Chen Q** (1989) The major low-molecular-weight heat-shock protein in chloroplasts show antigenic con-

servation among diverse plant species. Mol Cell Biol **9**: 461–468

29. **Werner-Washburne M, Stone DE, Craig EA** (1987) Complex interactions among members of an essential subfamily of *hsp* 70 genes in *Saccharomyces cerevisiae*. Mol Cell Biol **7**: 2568–2577

30. **Yamamori T, Yura, T** (1982) Genetic control of heat-shock protein synthesis and its bearing on growth and thermal resistance in *Escherichia coli* K-12. Proc Natl Acad Sci USA **79**: 860–864